Time of growth model for proteolytic *Clostridium botulinum*

A model was developed to predict the time for growth of 10^4 spores/tube of proteolytic strains of C. botulinum *in sealed tubes of broth media with varying pH (5–7), NaCl levels (0–3%) and storage temperatures (15–37°C). The proportion of positive samples from a set of inoculated tubes of an individual treatment combination were fitted to a logistic function having parameters for the time to turbidity, the rate at which tubes become turbid and the proportion of positive tubes after 62 days. These three parameters were then fitted to multiple regression equations where pH, NaCl levels and temperature were the descriptive parameters. Sodium chloride (<3%) had little effect on the time for growth, but temperatures below 20°C and pH values below 5·5 greatly delayed growth.*

Introduction

The hazard evaluation of a micro-organism such as *C. botulinum*, which originates from dormant spores and produces a potent neurotoxin, is more concerned with the germination-lag time than the rate of growth. Growth and toxin production are usually rapid compared to the time for germination and outgrowth. The potency of the toxin means any growth is unacceptable even though most of the toxin is not produced until the latter portion of the exponential growth phase (Siegel and Metzger 1979). Therefore, growth models such as the Gompertz (Gibson et al. 1987, Buchanan et al. 1989), square root (Ratkowsky et al. 1991) or kinetic-mechanistic (Baranyi et al. 1993, Whiting and Cygnarowicz-Provost 1992) are not appropriate.

Roberts et al. (1981) developed a probability model for botulinal toxin formation in a model pork slurry system as a function of various environmental and treatment factors. This model only estimated the probability of toxin being present within a 6 month period, but did not estimate at what time during this period it became toxic. Hauschild et al. (1982) added serially increasing numbers of spores to liver sausages and estimated the probability of toxin production from a single spore within a specified time period with use of the most probable number (MPN) tables (Oblinger and Koburger 1984). Baker et al. (1990) also utilized serial concentrations of spores and MPN tables to calculate the probabilities of spore outgrowth and toxin formation with storage time in fish. Samples were incubated at

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different temperatures and polynomial regression equations were calculated to describe probability of toxin formation as a function of temperature, inoculum and atmosphere.

These models provide an incomplete description of spore outgrowth and toxin production by not including the time of growth or toxin formation or they are based upon limited number of sample tubes, 3 or 5 MPN tubes for example, at the less favourable environmental conditions, which are most important conditions for modelling. This paper proposes a comprehensive primary model (Whiting and Buchanan 1993) that describes the accumulating number of positive samples with increasing storage time. The three parameters in the model provide information on time to turbidity, the rate at which samples become turbid, and the proportion of samples which never become turbid. Data for *C. botulinum* were fitted to the function and then multiple regression equations (a secondary model) were used to describe the effects of pH, NaCl level and temperature on the values of the function's parameters.

Materials and Methods

C. botulinum

A six strain mixture of three type A (69, FDA; 62, FDA; 33, U.S. Army Lab., Natick, MA, USA) and three proteolytic type B (169, FDA; 999, FDA; ATCC 7949) was prepared by growing each strain in BAM broth (Huhtanen 1975) at 35°C inside an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI, USA) for 3 weeks. Spores were centrifuged and washed with water and stored in water at 4°C. Purity of the spore preparations was determined by growing on BAM agar and observing the Gram strain and catalase reaction (negative). Plates incubated aerobically were negative. Each spore preparation was enumerated by serial dilutions, plating (Spiral Systems, Inc., Bethesda, MD, USA) and incubating (35°C) inside the chamber. Equal numbers of spores from each strain were combined to make an inoculating spore preparation of 10^5 spores ml^{-1}

Treatments

Sodium chloride (0–3% w/v) was added to aliquots of BAM broth and the pH (5–7) was adjusted with 0·1 N HCl. Culture tubes with 10·0 ml of broth were autoclaved and kept hot (*c.* 80°C) for inoculation with 0·1 ml of the spore mixture (10^4 spores $tube^{-1}$). Approximately 2–3 ml of melted, sterile VASPAR (45% paraffin, 55% white petroleum) was poured onto the broth. The spores were then heat shocked in 80°C water for 10 min. The tubes were air cooled without being disturbed so the VASPAR solidified into a plug on top of the broth. The tubes were incubated at 15, 20, 28 or 37°C and observed frequently over a 62 day period for signs of turbidity. The time for turbidity was recorded and an extra two days was then allocated to ensure sufficient toxin for detection. Samples were stored in the refrigerator for toxin confirmation by enzyme-linked immunoassay procedures (ELISA) (Huhtanen et al. 1992). Rapid growth conditions had a minimum of six tubes per treatment combination, slow growth conditions had up to 28 tubes per treatment. The total number of inoculated tubes in 28 treatments was 463.

Modelling

The probability of growth was calculated as the number of tubes showing signs of growth in each treatment combination divided by the total number of tubes in that treatment. The probability of growth as a function of storage time was fitted to the following function by the RS/1 statistical program (BBN Software Products Corp, Cambridge, MA, USA).

$$P_{(t)} = P_{max}/(1 + e^{k(\tau - t)}) \quad (1)$$

$P_{(t)}$, probability of growth at t;
t, time (days);
P_{max}, maximum probability of growth after 62 days;
k, rate constant ($days^{-1}$);
τ, time of the midpoint of the function (days).

$P_{(t)}$ initially had a value of 0·0 and can increase to 1·0 if all the replicate tubes have growth. The τ value is the time for half of the replicates that will become toxic to show turbidity. The values for P_{max}, k and τ were de-

Table 1. Time for turbidity in selected treatments.

Treatment				
Temp (°C)	pH	NaCl (%)	Total number of tubes	Time each tube became turbid (days)
20	7	3	15	6 6 6 6 6 7 7 7 8 8 10 10 12 17 —
20	5·5	0·75	18	6 16 16 19 24 31 31 34 — — — — — — — — — —
20	5	0	19	50 52 55 55 57 59 — — — — — — — — — — — — —

—, Tube did not become turbid within 62 days.

termined for each of the 28 treatment combinations. Regression procedures for the effect of the descriptive parameters (pH, NaCl and temperature) on each of the regressed parameters (P_{max}, k and τ) was performed to find the best equation using RS/1. The function was solved for t with $P_{(t)}$ set to 0·1. This gave the estimated time for 10% of the samples to show growth.

Results

To illustrate the general patterns of increasing numbers of positive tubes with storage, data from three of the 28 treatment combinations are shown in Table 1. The first combination, a favourable condition, had the first turbid tubes after 6 days. Additional tubes had growth through day 17; the one remaining tube did not become turbid for the remaining storage period. The second treatment also had the first tube become turbid on day 6, but additional growth was slower and only 8 of 18 tubes had growth after 62 days. The last treatment combination was least favourable, the first turbidity occurred on day 50 and only 6 of 19 tubes had growth after 62 days. Figure 1 shows the graph of the probability of growth with time for these three treatment combinations. The toxin assays established that the turbid samples contain toxin and the tubes clear after 62 days did not. The ELISA was sensitive to approximately 10 mouse units of toxin/ml.

The fits of the primary model to each of the treatment combinations and the

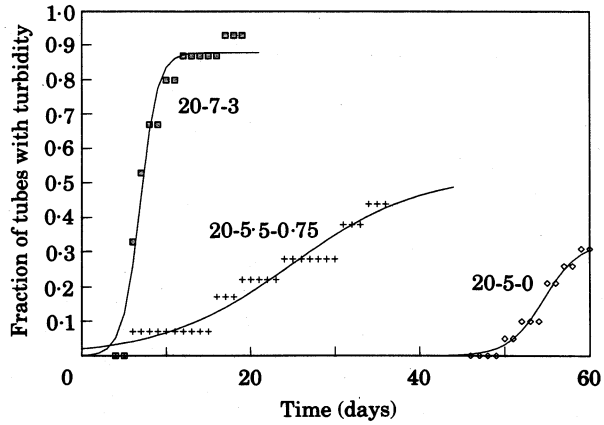

Fig. 1. Fraction of positive samples with time for selected treatment combinations. Designations are temperature, pH and NaCl levels, respectively. Symbols represent data, the line represents the fit of that data to the model (Eqn 1).

Table 2. Fitted and calculated values for P_{max}, k and τ at each treatment combination.

Temperature (°C)	pH	NaCl (%)	Observed values						Calculated values			Time for $P_{(t)} = 0{\cdot}1$
			P_{max}	s.e. P_{max}	k	s.e. k	τ	s.e. τ	P_{max}	k	τ	
35	7·0	0·00	0·91	0·33	4·6	4·8	1·2	0·6	1·03	4·8	1·2	0·7
35	7·0	3·00	0·91	0·33	4·6	4·8	1·2	0·6	0·88	3·9	1·3	0·8
35	6·0	0·00	0·84	0·01	3·9	0·4	2·6	0·1	0·78	3·7	2·0	1·5
35	6·0	1·50	0·84	0·04	3·1	0·6	2·2	0·1	0·86	3·3	2·1	1·5
35	5·5	0·75	0·93	0·02	4·9	0·7	1·6	0·1	0·74	2·8	3·0	2·3
35	5·0	0·00	0·57	0·02	0·8	0·1	6·9	0·2	0·55	2·3	4·5	3·9
35	5·0	3·00	0·51	0·04	0·6	0·1	4·5	0·4	0·54	1·5	4·8	3·8
28	7·0	0·00	1·00	0·22	1·9	0·7	2·2	0·6	1·12	2·5	1·8	0·9
28	7·0	3·00	0·90	0·03	1·1	0·3	3·3	0·2	0·94	1·6	2·3	1·0
28	6·0	0·00	0·99	0·13	0·4	0·2	3·3	1·0	0·83	1·9	3·3	2·2
28	6·0	1·50	1·00	0·09	0·6	0·1	4·2	0·5	0·88	1·5	3·6	2·2
28	5·5	0·75	0·64	0·01	0·6	0·1	3·6	0·3	0·75	1·3	5·1	3·6
28	5·0	0·00	0·34	0·01	4·1	0·4	5·5	0·1	0·55	1·1	7·6	6·3
28	5·0	3·00	0·35	0·01	0·5	0·1	11·7	0·5	0·50	0·2	8·8	2·3
20	7·0	0·00	1·00	0·02	2·2	0·3	4·3	0·1	0·96	1·4	6·2	4·7
20	7·0	3·00	0·88	0·02	1·0	0·1	6·9	0·2	0·75	0·5	8·5	4·9
20	6·0	0·00	0·64	0·03	3·3	0·6	5·6	0·1	0·62	1·4	11·5	10·3
20	6·0	1·50	0·79	0·04	0·1	0·0	15·8	0·1	0·66	1·0	13·2	11·5
20	5·5	0·75	0·53	0·06	0·1	0·0	24·9	2·2	0·51	1·2	18·5	17·3
20	5·0	0·00	0·33	0·02	0·5	0·1	54·6	0·4	0·29	1·3	27·9	27·3
20	5·0	3·00	0·28	0·01	0·9	0·2	28·3	0·3	0·21	0·4	35·1	34·8
15	7·0	0·00	0·93	0·01	0·2	0·0	24·1	0·3	0·73	1·6	19·4	18·2
15	7·0	3·00	0·35	0·10	0·4	0·1	25·6	1·6	0·49	0·7	28·0	26·1
15	6·0	0·00	0·12	0·02	4·0	2·2	56·3	0·2	0·36	2·0	36·6	36·2
15	6·0	1·50	0·26	0·03	2·8	1·2	49·8	0·3	0·38	1·6	43·4	42·8
15	5·5	0·75	0·00		0·0[a]				0·22	2·0	60·5	60·4
15	5·0	0·00	0·20	0·05	0·4	0·2	54·3	2·1	0·00	2·2	91·0	[b]
15	5·0	3·00	0·00		0·0[a]				–0·11	1·4	121·1	[b]

[a]No growth in any tube in this treatment.
[b]No growth predicted.

standard error for each parameter are given on Table 2. The large standard errors for the first two combinations resulted from all the replicate tubes becoming turbid after one day, the absence of the intermediate data points gave a high standard error. With temperature, pH and NaCl level the descriptive variables, regression equations for the parameters were calculated (secondary model) (Table 3).

The primary model function Eqn (1) can be rearranged to calculate the time for the probability to reach a particular value.

$$t = \tau - (2{\cdot}31\log((P_{max}/P_{(t)}) - 1)/k) \quad (2)$$

The times for $P_{(t)} = 0{\cdot}1$ are calculated for each of the 28 treatments using the appropriate values of P_{max}, k and τ (Table 2).

Discussion

Nordin et al. (1975) described the pattern of growth from *C. botulinum* spores as having a lag period then a rapid outgrowth followed by no additional outgrowth. Graphic representations were presented by Ivey and Robach (1978), Tompkin (1978a, 1979), Sofos et al. (1980) and Sofos (1985). The primary model describes the pattern of observa-

Table 3. Regression coefficients and their significance for the effect of descriptive parameters (temperature, pH and % NaCl) on model parameters (P_{max}, k, τ).

	P_{max}		k[a]		$\log_{10}\tau$	
Term	Coefficient	Significance	Coefficient	Significance	Coefficient	Significance
C	–3·613	0·33	12·36	0·05	8·156	0·07
Temp	0·1655	0·00	–1·058	0·03	–0·2004	0·01
pH	0·3831	0·75			–1·0774	0·45
NaCl	0·2063	0·38	–0·288	0·21	0·0397	0·89
Temp × pH	–0·00622	0·24	0·0785	0·11	0·0020	0·77
Temp × NaCl	0·00151	0·64			–0·0016	0·70
pH × NaCl	–0·0222	0·42			0·0059	0·86
$Temp^2$	–0·00213	0·01	0·0134	0·06	0·0025	0·01
pH^2	0·00630	0·94	–0·126	0·24	0·0593	0·61
$NaCl^2$	–0·0510	0·30			–0·0012	0·98
F value		10·7**		3·2*		21·1**
R^2		0·84		0·44		0·92

[a]Missing coefficients were deleted during backwards elimination of regression terms.
*Significant at the 95% level.
**Significant at higher than 99% level.

tions of a time for growth experiment in these papers more completely than does a mean or another way of expressing the data. The number of replicate tubes for a particular treatment combination can be increased for additional precision if P_{max} is small or if accurate estimates of times for growth at low $P_{(t)}$ values are desired.

Inferences to the behaviour of a single spore are not directly possible with the data in this paper. Having several inoculation levels would provide an estimate of the behaviour of low numbers of spores found in most foods by allowing inocula number to be an additional factor in the regression equation. The probability of growth from single spore models based upon MPN designs (Baker et al. 1990) do not provide more precise information because the conditions of slow or limited growth typically rely on the data from only three tubes each of two serial dilutions with high inocula that have growth.

P_{max} and log τ had significant regression coefficients ($P \leq 0{\cdot}01$) and R^2 values of 0·84 and 0·92, respectively. As is typical for time parameters, the regression equation for log τ was better than for τ, F values were 21·1 and 12·4, respectively. The complete second-order regression equation was used to calculate the values for P_{max} and τ on Table 2, even though only the temperature terms were significant. The slope (k) was not consistently affected by temperature, pH or NaCl levels and the complete regression equation was not significant ($P > 0{\cdot}05$). Therefore, backwards elimination of terms was performed until a significant regression equation was obtained that did not calculate any negative values for k. This equation still had a low R^2 of 0·44. Comparison of observed values for P_{max} and τ with those calculated by the regression equations showed good agreement. The combination of 20°C–pH 5–0% NaCl had the worst agreement for τ. This appeared to be an anomaly of the data rather than the regression equations. The calculated P_{max} did have values greater than 1·0 at two rapid growth conditions and a value

less than 0·0 at a non-growth condition, but these do not greatly affect the use of the model. The calculated τ values for the last three conditions exceed 60 days and should be interpreted as > 60 days. A spreadsheet was created to take entered values of temperature, pH and NaCl levels and calculate P_{max}, k and τ. These parameters were then used to solve the primary model (Eqn 1) for the probability of growth at each day of storage which was displayed as a graph. This showed that temperature and pH are more important than NaCl (≤3%) in controlling growth. Times for growth are greatly increased and the maximum probabilities of growth are decreased when the temperature is less than 20°C or the pH below 5·5.

The calculation of the time to reach a probability of 0·1 provides an estimate of when the first samples were likely to become toxic. This value may be more important to users of the model than the usual calculation of the time for half the samples to become toxic.

The primary model presented in this paper is generally applicable for similar time-to-growth situations. The secondary model (regression equations) are descriptive of the data set in this paper. The regression equations are least squares fits and not intended to be the minimum or 'fail-safe' times for growth. These equations have not been validated against data from *C. botulinum* growth in foods and should be used with caution.

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